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A polymorphic bipartite motif signals nuclear targeting of early auxin-inducible proteins related to PS-IAA4 from pea (*Pisum sativum*)

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Summary

The plant hormone, indoleacetic acid (IAA), transcriptionally activates two early genes in pea, *PS-IAA4/5* and *PS-IAA6*, that encode short-lived nuclear proteins. The identification of the nuclear localization signals (NLS) in PS-IAA4 and PS-IAA6 using progressive deletion analysis and site-directed mutagenesis is reported. A C-terminal SV40-type NLS is sufficient to direct the β-glucuronidase reporter to the nucleus of transiently transformed tobacco protoplasts, but is dispensible for nuclear localization of both proteins. The dominant and essential NLS in PS-IAA4 and PS-IAA6 overlap with a bipartite basic motif which is polymorphic and conserved in related proteins from other plant species, having the consensus sequence (KKNEK)KR-$X_{(24-71)}$-(RSXRK)/(RK/RK). Both basic elements of this motif in PS-IAA4, (KR-X_{41}-RSYRK), function interdependently as a bipartite NLS. However, in PS-IAA6 (KKNEKKR-X_{36}-RKK) the upstream element of the corresponding motif contains additional basic residues which allow its autonomous function as an SV40-type monopartite NLS. The spacer-length polymorphism, $X_{(24-70)}$, in respective bipartite NLS peptides of several PS-IAA4-like proteins from *Arabidopsis thaliana* does not affect nuclear targeting function. The structural and functional variation of the bipartite basic motif in PS-IAA4-like proteins supports the proposed integrated consensus of NLS.

Introduction

The plant growth hormone auxin, typified by indole-3-acetic acid (IAA), regulates various aspects of plant development by affecting fundamental processes of plant cell growth and function, such as cell elongation, cell division, ion transport, and differentiation (Estelle, 1992; Went and Thimann, 1937). Auxin-mediated cell elongation is one of the fastest hormonal responses known (Evans and Ray, 1969). At the molecular level, auxin exerts its regulatory role by modulating membrane function (Assman, 1993; Blatt and Thiel, 1993) and gene expression (Guilfoyle, 1986; Theologis, 1986). However, the molecular mechanism(s) of auxin action are not understood.

Auxin-mediated cell elongation is associated with rapid changes in the expression of a select set of primary genes (Guilfoyle, 1986; Theologis, 1986). Although several primary auxin-responsive genes have been isolated and structurally characterized, the signaling events involved in their transcriptional activation and the biochemical function of their encoded proteins are unknown (Estelle, 1992; Hobbie *et al.*, 1994). Two genes from pea, *PS-IAA4/5* and *PS-IAA6*, are rapidly (within 4–8 min) induced by auxin (Theologis *et al.*, 1985). The induction by IAA is due to transcriptional activation and does not involve stabilization of the labile transcripts (Koshiba and Theologis, unpublished data). Both genes have been structurally characterized, and the auxin-responsive region of the *PS-IAA4/5* promoter has been functionally identified (Ballas *et al.*, 1993; Oeller *et al.*, 1993). The encoded proteins, PS-IAA4 and PS-IAA6, share similar physical properties and extensive amino acid sequence identity in four domains which are conserved in polypeptides encoded by related early auxin-inducible genes, *Aux22* and *Aux28* from soybean (Ainley *et al.*, 1988), *ARG3* and *ARG4* from mung bean (Yamamoto *et al.*, 1992), *AtAux2-11* and *AtAux2-27* (Conner *et al.*, 1990), *IAA1* and *IAA2* (Abel and Theologis, 1994) and *IAA3-IAA14* (Abel and Theologis, unpublished data) from *Arabidopsis*. A conspicuous structural feature of PS-IAA4-like proteins is a conserved putative βαα-motif with similarity to the β-ribbon DNA-binding domain of prokaryotic repressor proteins (Abel *et al.*, 1994; Raumann *et al.*, 1994). Both polypeptides are short-lived, translationally regulated, and can direct, as fusions, a β-glucuronidase (GUS) reporter protein into the cell nucleus (Abel *et al.*, 1994; Oeller and Theologis, 1995). Thus, an anticipated nuclear function of these proteins appears to be primarily regulated by rapid changes of steady-state gene expression. The data thus far obtained suggest a regulatory function of PS-IAA4-like proteins.

The importance of nuclear transport as an additional major regulatory mechanism to control nuclear protein function has been increasingly demonstrated for transcriptional activators which act as key genetic switches in development, cell division-cycle and signal transduction pathways (Forbes, 1992; Garcia-Bustos *et al.*, 1991; Hill and Treisman, 1995; Hunter and Karin, 1992). Nuclear protein import involves selective targeting to and energy-dependent translocation through the nuclear pore complex. Pro-

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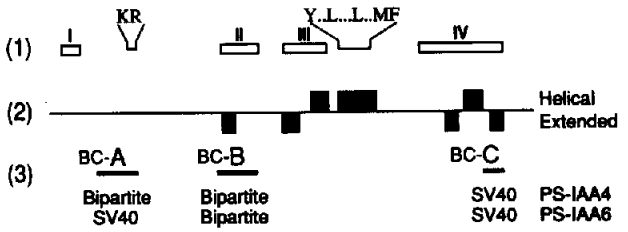


Figure 1. Domain structure of PS-IAA4 and PS-IAA6.
The following are shown schematically: (1) conserved domains (I–IV) and invariant amino acids in the variable regions of the primary structure; (2) conserved secondary structure elements as predicted (approximately 70% prediction accuracy) by a neural network algorithm (Rost and Sander, 1993); and (3) positions of conserved basic clusters (BC), A, B, and C, basic amino acids of which are part of putative NLS sequences (PS-IAA4: BC-A, KKIIHGSSVVKNNNKR[36]; BC-B, KAKIVGWPPIRSYRK[82]; BC-C, KRLRIMK[178]; PS-IAA6: BC-A, KKNEKKR[29]; BC-B, KKNQVVGWPPVCSYRKK[68]; BC-C, KRLRIMK[159]; see Abel *et al.*, 1994).

teins that contain nuclear localization signal (NLS) sequences are complexed by receptors which specifically recognize NLS, and are targeted to the nuclear pore complex with the aid of auxiliary factors (Goldfarb, 1994). Nuclear targeting signals are integral parts of nuclear protein structure and consist usually of short sequences with a high proportion of basic amino acids. Depending on the structure and amino acid composition, these basic clusters function either independently as monopartite NLS or interdependently as bipartite NLS (Dingwall and Laskey, 1991; Raikhel, 1992). Mechanisms to control nuclear targeting involve regulation of (multiple) NLS accessibility by ligand binding or protein interaction and modulation of NLS activity by biochemical modification (Forbes, 1992; Goldfarb, 1994; Hunter and Karin, 1992).

In a continuing effort to elucidate the functional domains and the regulation of PS-IAA4-like proteins, we have analyzed the nuclear targeting signals in PS-IAA4 and PS-IAA6. The comparative analysis reveals that the dominant and necessary NLS in both proteins overlaps with a bipartite basic motif which is conserved but polymorphic in related polypeptides. The polymorphism affects amino acid composition of the two basic elements and size of the intervening region. The structural diversity and functional flexibility of the bipartite basic motif conserved in this class of ubiquitous auxin-regulated proteins directly supports the integrated consensus of NLS proposed by Dingwall and Laskey (1991).

Results

Strategy to identify functional NLS in PS-IAA4 and PS-IAA6

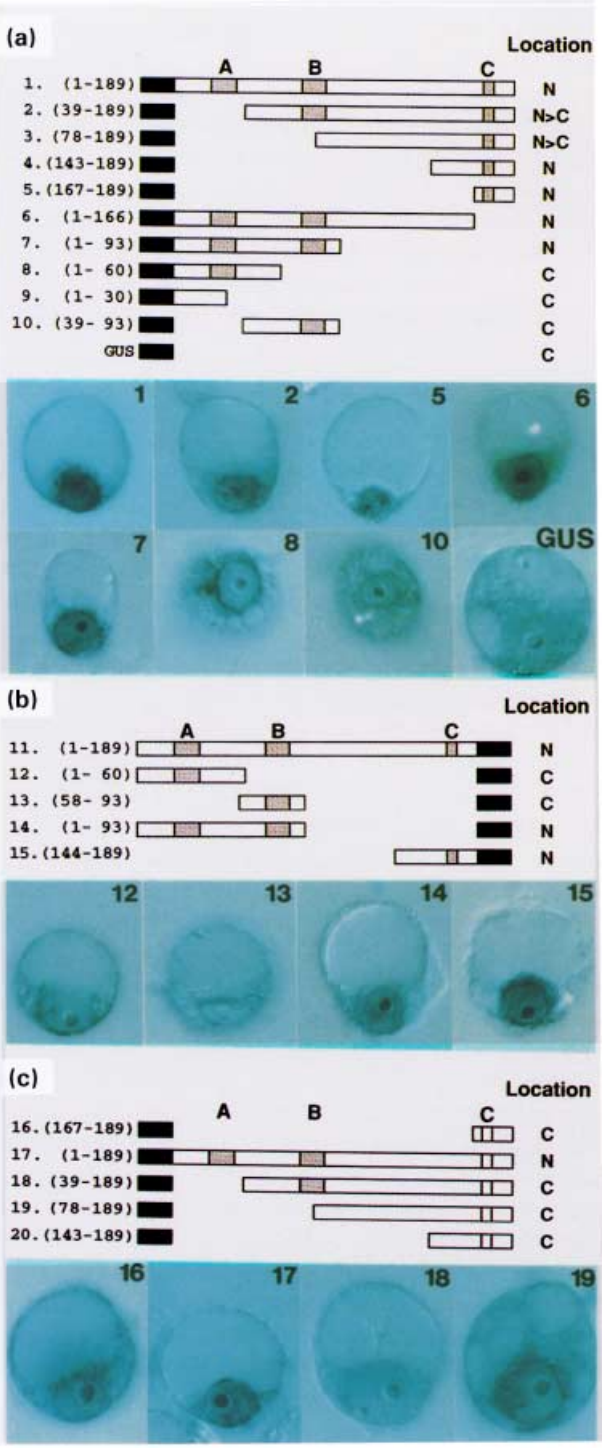
Analysis of the primary structure of PS-IAA4-like proteins reveals three conserved clusters of basic amino acids which satisfy structural requirements of three major classes of NLS (Abel *et al.*, 1994). As outlined in Figure 1 for PS-IAA4 and PS-IAA6 from pea, a putative NLS sequence with similarity to the SV40 large T antigen NLS (PKKKRKV[132] (Kalderon *et al.*, 1984); smallest consensus: KR/KXR/K (Chelsky *et al.*, 1989)) is found at the end of conserved domain IV in basic cluster (BC) BC-C. This sequence (KRLRIMK in both proteins) also appears similar to the MATα2-like NLS (KIPIK[7] (Hall *et al.*, 1984)). A motif composed of two basic elements separated by a short spacer (KAK-X_7-RSYRK[82] in PS-IAA4 and KK-X_{12}-RKK[68] in PS-IAA6), BC-B, constitutes conserved domain II and resembles the prototypical bipartite NLS in nucleoplasmin from *Xenopus* (KR-X_{10}-KKKK[170] (Robbins *et al.*, 1991)) and bipartite NLS sequences in plant nuclear proteins (Raikhel, 1992). The two invariant basic amino acids, KR, in the variable region between conserved domain I and domain II, BC-A, are proposed to be part of a potential bipartite NLS (KK-X_8-KNNNKR[36]) in PS-IAA4 and of a potential SV40-type NLS (KKNEKKR[29]) in PS-IAA6, respectively.

Using the experimental system of Restrepo *et al.* (1990), we have previously demonstrated nuclear localization of PS-IAA4 and PS-IAA6 in transiently transfected tobacco protoplasts (Abel *et al.*, 1994). The nuclear accumulation of GUS::PS-IAA4 and GUS::PS-IAA6 fusion proteins in pea protoplasts confirms these results in a homologous transformation system (data not shown). Progressive deletion analyses and site-directed mutagenesis have been employed to analyze functional NLS in PS-IAA4 and PS-IAA6. Since the configuration of GUS chimera may affect nuclear targeting efficiency (Shieh *et al.*, 1993; Tinland *et al.*, 1992; Varagona *et al.*, 1991), most informative constructs of deletion analyses were tested both as N-terminal and as C-terminal fusions with GUS. To minimize the possibility of artifactual folding of mutant proteins, potential NLS sequences were mutagenized by introducing non-conservative mutations which were designed to maintain the predicted secondary structure (Rost and Sander, 1993) of PS-IAA4 and PS-IAA6 polypeptides (see Figure 1).

Deletion mapping of PS-IAA4 reveals two independent NLS

Since the putative NLS sequences in the three conserved clusters of basic amino acids (BC-A, BC-B, BC-C, Figure 1) may not necessarily coincide with the functional karyophilic signals, we first studied the effect of progressive N-terminal and C-terminal deletions of PS-IAA4 on the nuclear localization of GUS::PS-IAA4 fusion proteins. A series of deletions from the N-terminus reveals that a short C-terminal region (amino acids 167–189, FVTSCKRLRIMKGTEAKGLGCGV[189]) is sufficient to localize GUS to the nucleus (Figure 2a, construct 5). This sequence contains the putative SV40-type NLS motif KRLRIMK[178] (BC-C). Interestingly, PS-IAA4 deleted for the C-terminus still mediates efficient nuclear localization of GUS (Figure 2a, construct 6, amino acids 1–

166). Analysis of progressive C-terminal deletions (Figure 2a, constructs 6–9) indicates the presence of a second karyophilic signal in a central region encompassing BC-B (Figure 2a, compare construct 7 with 8). Overlapping fragments derived from the N-terminal half of PS-IAA4 which comprise either BC-A (Figure 2a, construct 8, aa 1–60) or BC-B (Figure 2b, construct 10, amino acids 39–93) were individually tested. However, unlike the parental fragment (Figure 2a, construct 7, amino acids 1–93), both deletion derivatives are insufficient to direct GUS to the nucleus (Figure 2a, compare construct 7 with 8 and 10). This result suggests the presence of a bipartite motif promoting nuclear localization which requires N-terminal and central sequences of PS-IAA4.



To further support these observations and to minimize the probability that an NLS escapes detection due to improper folding of GUS hybrids, selected deletions were additionally tested as N-terminal reporter fusions (Figure 2b). Similar results were obtained as compared with corresponding C-terminal GUS fusions (Figure 2a and b, compare constructs 8 with 12, 10 with 13, 7 with 14, and 4 with 15). These results indicate the presence of two NLS in PS-IAA4, an N-terminal bipartite signal (NLS-N), and a C-terminal signal (NLS-C), which are individually sufficient to direct GUS to the nucleus.

The effect of multiple point mutations aimed at basic amino acids in BC-C (KRLRIMK^{178} → TGLSIMT^{178}) was studied to further characterize NLS-C. Unlike the C-terminal peptide of wild-type PS-IAA4 (Figure 2a, construct 5, amino acids 167–189), the mutant peptide does not promote nuclear localization of GUS (compare construct 5 in Figure 2a with construct 16 in Figure 2c). The elimination of nuclear import indicates that the basic amino acids of the SV40-type sequence KRLRIMK^{178} are part of NLS-C in PS-IAA4. However, introduction of the same point mutations into full-length PS-IAA4 has no effect on nuclear targeting (Figure 2c, construct 17). This observation and the analysis of progressive N-terminal deletions of PS-IAA4 containing a mutant NLS-C are consistent with the existence of two independent NLS (Figure 2c). The deletion analyses in Figure 2 also indicate that the putative nuclear targeting signals in BC-A and BC-B are individually not sufficient to promote nuclear localization but, instead, may compose the bipartite structure of NLS-N.

Bipartite NLS-N is the essential karyophilic signal in PS-IAA4

In a second approach, we wished to probe more directly the role of the two karyophilic signals in nuclear transport

Figure 2. Deletion mapping of NLS in PS-IAA4.
C-terminal (a and c) and N-terminal (b) fusions between PS-IAA4 derivatives (open box) and GUS (black box, not to scale) are shown schematically. Positions of conserved basic clusters are marked by stippled boxes (designated as A, B, and C; see Figure 1); multiple point mutations in BC-C (KRLRIMK^{178} → TGLSIMT^{178}) are indicated by an open box. Amino acid designations of PS-IAA4 derivatives fused to the GUS protein are given in parentheses on the left for each plasmid construct. Constructs are numbered consecutively throughout Figures 2, 3 and 6. The subcellular localization of the chimeric GUS proteins in transformed tobacco protoplasts is indicated on the right (N, nuclear; C, cytoplasmic; N>C, predominantly nuclear). Histochemical GUS staining micrographs are shown for selected transfections at the bottom of each panel.

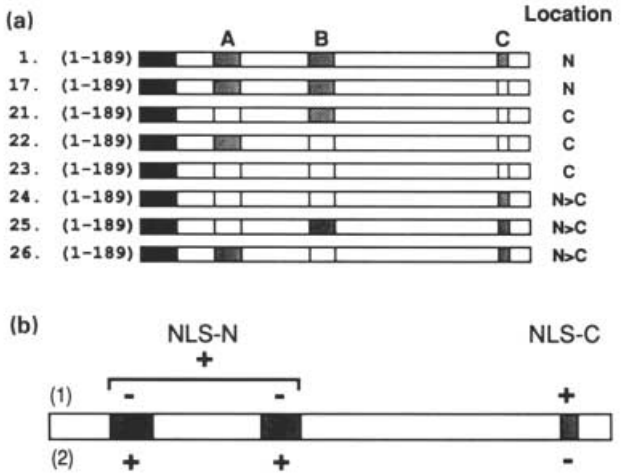


Figure 3. Mutagenesis analysis of NLS in PS-IAA4.
(a) Effect of mutagenized basic clusters on the subcellular localization of GUS::PS-IAA4 fusion proteins. Positions of conserved basic clusters are marked by stippled boxes (A–C); mutations therein are indicated by an open box (BC-A, KKIIHGSSVVKNNNKR^{36}→ENIIHGSSVLENNNES^{36}; BC-B, KAKIVGWPPIRSYRK^{82}→PAVIVGWPPICSYGT^{82}; BC-C, KRLRIMK^{178}→TGLSIMT^{178}).
(b) Schematic summary of NLS analysis in PS-IAA4. (1) denotes basic clusters (stippled boxes) which are sufficient (+) to direct GUS to the nucleus as inferred from deletion analysis (see Figure 2). (2) denotes basic clusters which are necessary (+) for efficient nuclear transport of the full-length protein fused to GUS as inferred from site-directed mutagenesis (this figure).

of the full-length PS-IAA4 protein. To test whether the putative signal sequences in BC-A and BC-B are part of NLS-N and to define whether NLS-N and NLS-C (BC-C) are functional and necessary for nuclear localization of PS-IAA4, we used site-directed mutagenesis to replace simultaneously multiple basic amino acids within these clusters. The resulting permutation matrix of mutagenized basic clusters in GUS::PS-IAA4 fusion proteins is shown in Figure 3(a).

Mutation of BC-C alone does not visibly affect the efficiency of nuclear transport which implies that NLS-C is not an essential karyophilic signal in PS-IAA4 (Figure 3a, compare construct 1 with 17). However, additional mutation of potential NLS sequences either in BC-A or BC-B (or in both) prevent nuclear accumulation of the respective GUS chimera (Figure 3a, compare construct 17 with 21–23). These data clearly show that the basic amino acids in BC-A and BC-B constitute bipartite NLS-N and that both basic clusters function in an interdependent manner. The inhibition of nuclear localization also indicates that, most likely, all karyophilic signals in PS-IAA4 have been inactivated by mutagenesis though masking of additional NLS caused by improper folding of chimeric GUS proteins cannot be ruled out.

In context with wild-type NLS-C, mutation of the bipartite structure (BC-A and BC-B) results in significant cytoplasmic localization of GUS activity (Figure 3a, compare construct 1 with 24). However, the histochemical staining is visibly more intense in the nucleus than in the cytoplasm. Essentially, the same degree of subcellular partitioning of GUS activity is observed for GUS::PS-IAA4 fusion proteins containing mutations in either BC-A or BC-B (Figure 3a, compare construct 24 with 25 and 26). The partitioned subcellular localization of GUS activity for PS-IAA4 fusions defective in the bipartite karyophilic signal is comparable to the subcellular distribution of GUS activity for deletion mutants of PS-IAA4 void of an intact bipartite signal (compare constructs 24–26 in Figure 3a with constructs 2 and 3 in Figure 2a). Although NLS-C, as an isolated entity, proves to be highly sufficient to mediate nuclear localization of GUS, its targeting ability is significantly reduced in a more comprehensive native protein context. This phenomenon is not uncommon and has recently been described for the C-terminal NLS of the R protein from maize (Shieh *et al.*, 1993). The functional analysis of karyophilic signals in PS-IAA4 is schematically summarized in Figure 3(b).

Comparative analysis reveals similar NLS in PS-IAA6

Similar clusters of basic amino acids which compose bipartite NLS-N and SV40-type NLS-C in PS-IAA4, are conserved in the related PS-IAA6 polypeptide. Therefore, a comparative analysis of NLS in PS-IAA6 is expected to substantiate the observations for PS-IAA4, although BC-A in PS-IAA6 (KKNEKKR^{29}) differs significantly in sequence from the respective cluster in PS-IAA4 (KKIIHGSSVVKNNNKR^{36}), except for the two invariant C-terminal basic amino acids, KR (see Figure 1).

A series of N-terminal deletions (Figure 4a, constructs 1–4) reveal that the C-terminus of PS-IAA6 (amino acids 142–179) which also contains the SV40-type NLS sequence KRLRIMK^{159}, is less effective in localizing GUS to the nucleus as the respective region of PS-IAA4 containing NLS-C. Substantial nuclear localization mediated by the C-terminus of PS-IAA6 is only detectable for N-terminal GUS fusions (compare constructs 3 and 4 in Figure 4a with 15 in Figure 4b). Deletion of the N-terminus of PS-IAA6 (amino acids 1–35), which contains the potential SV40-type NLS sequence KKNEKKR^{29} in BC-A, completely prevents nuclear localization of the respective GUS hybrid (Figure 4a, compare construct 1 with 2). This is consistent with the assumption that basic amino acids of BC-A and BC-B constitute, as in PS-IAA4, a bipartite karyophilic signal. However, progressive C-terminal deletions indicate a functional autonomy of the first basic cluster (BC-A) of the anticipated bipartite element. Irrespective of the configuration of GUS fusion proteins, N-terminal sequences of PS-IAA6 (amino acids 1–84; or amino acids 1–45, MAREGLGLEITELRLGLSCGEPKKNEKKRMFSEIDGGVEENGGSG^{45}) are sufficient to promote nuclear localization of GUS (compare construct 6 in Figure 4a with construct 14 in Figure 4b, and construct

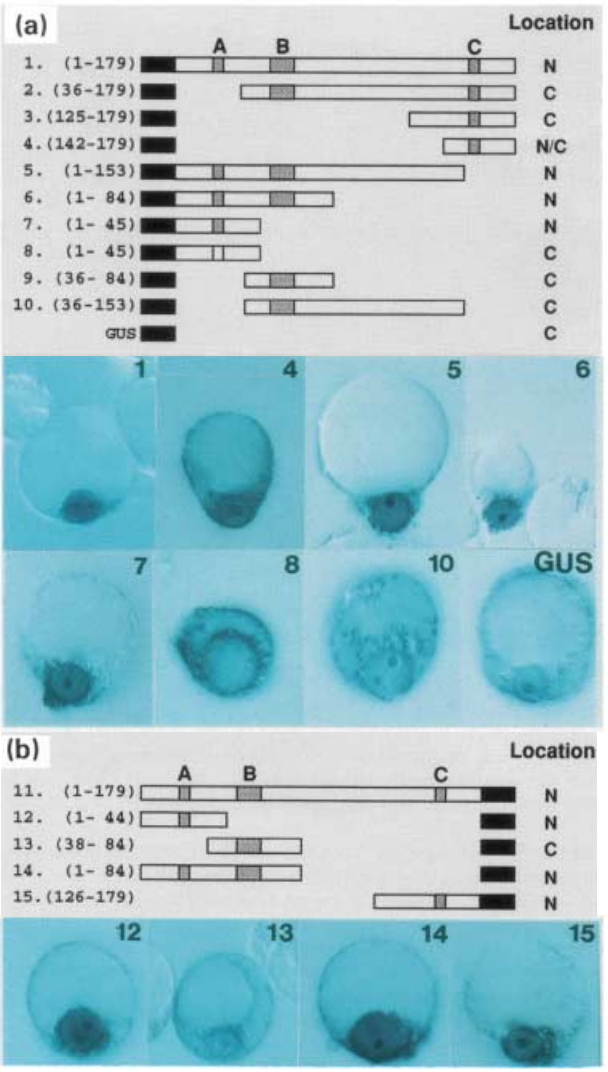


Figure 4. Deletion mapping of NLS in PS-IAA6.
C-terminal (a) and N-terminal (b) fusions of PS-IAA6 derivatives (open box) and GUS (black box, not to scale) are shown schematically. Positions of conserved basic clusters are marked by stippled boxes (designated as A, B, and C; see Figure 1); multiple point mutations in BC-A (KKNEKKR^{29}→ VNNENKL^{29}) are indicated by an open box.

7 in Figure 4a with construct 12 in Figure 4b). Internal polypeptides of PS-IAA6 encompassing BC-B are not sufficient to direct GUS to the nucleus (constructs 9, 10 in Figure 4a and 13 in Figure 4b).

To locate the N-terminal karyophilic signal, the effect of multiple point mutations in the potential SV40-type NLS (KKNEKKR^{29}→ VNNENKL^{29}) was studied. Unlike the N-terminal peptide (amino acids 1–45) of wild-type PS-IAA6, a corresponding mutant peptide does not promote nuclear accumulation of GUS (Figure 4a, compare construct 7 with 8), thereby defining the SV40-type NLS sequence KKNEKKR^{29} as part of the N-terminal karyophilic signal in PS-IAA6. Next, we assessed by site-directed mutagenesis the relevance of the three conserved basic clusters for

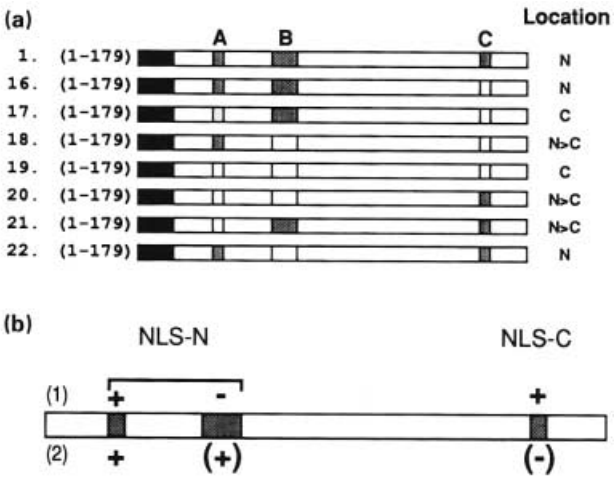


Figure 5. Mutagenesis analysis of NLS in PS-IAA6.
(a) Effect of mutagenized basic clusters on the subcellular localization of GUS::PS-IAA6 fusion proteins. Positions of conserved basic clusters are marked by stippled boxes (A–C); mutations therein are indicated by an open box (BC-A, KKNEKKR^{29}→VNNENKL^{29}; BC-B, KKNQVVGWPPVCSYRKK^{88}→AVNQVVGWPPVCSYGTN^{88}; BC-C, KRLRIMK^{159}→TGLSIMT^{159}).
(b) Schematic summary of NLS analysis in PS-IAA6. (1) denotes basic clusters (stippled boxes) which are sufficient (+) to direct GUS to the nucleus as inferred from deletion analysis (see Figure 4). (2) denotes basic clusters which are necessary (+) for efficient nuclear transport of the full-length protein fused to GUS as inferred from site directed mutagenesis (this figure). It should be pointed out that the mutagenesis analysis revealed that BC-B or BC-C are necessary for efficient nuclear transport only in the presence of BC-A and thus are shown in parentheses.

nuclear transport of full-length PS-IAA6. The respective permutation matrix is shown in Figure 5(a). As compared with wild-type PS-IAA6, mutation of BC-C alone does not visibly alter the efficiency of nuclear localization (Figure 5a, compare construct 1 with 16) which implies that NLS-C, as in PS-IAA4, is not an essential karyophilic signal in PS-IAA6. Additional mutation of BC-A abolishes nuclear localization, whereas mutation of BC-C and BC-B still allows substantial nuclear localization of GUS, although to a reduced extent (Figure 5a, compare construct 16 with 17 and 18). These observations suggest relatively independent function of BC-A that, however, requires synergistic action of basic residues of BC-B for efficient targeting activity. As expected, mutation of both terminal SV40-type NLS sequences (BC-A and BC-C) or of all three basic clusters completely prevents nuclear transport of the respective GUS::PS-IAA6 mutant proteins (Figure 5a, constructs 17 and 19). Mutation of the bipartite structure (BC-A and BC-B) or of its first basic cluster reveals the limited role in nuclear transport of NLS-C (BC-C) which is concluded from the subcellular partitioning of GUS activity (Figure 5a, compare construct 1 with 20 and 21). However, mutation of BC-B has no effect on the exclusive nuclear localization of GUS activity (Figure 5a, compare construct 1 with 22) which indicates that both SV40-type transport signals (BC-A and BC-C) act additively in nuclear targeting of PS-IAA6

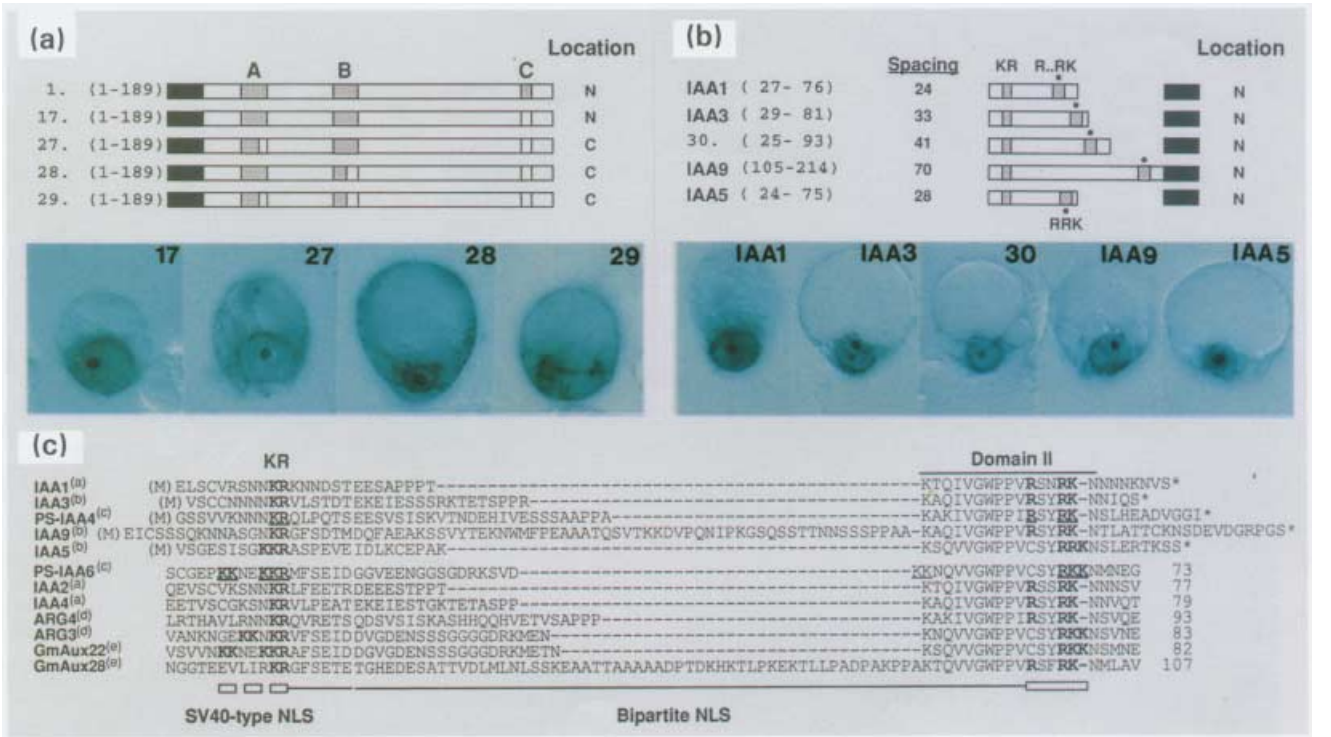


Figure 6. Primary structure of the bipartite basic motif in PS-IAA4-like proteins.

(a) Effect of mutations of invariant basic amino acids in PS-IAA4-like proteins on the subcellular localization of GUS::PS-IAA4 fusion proteins. Positions of conserved basic clusters are marked by stippled boxes (A–C), mutations of invariant basic amino acids in BC-A and BC-B (BC-A, KKIIHGSSVVKNNNKR^{36}→KKIIHGSSVVKNNNLE^{36}; BC-B, KAKIVGWPPIRSYRK^{82}→KAKIVGWPPICSYGT^{82}), and mutation of BC-C (KRLRIMK^{178}→TGLSIMT^{178}) are indicated by open boxes.

(b) Spacer-length polymorphism of the bipartite basic motif in PS-IAA4-like proteins. Internal peptides of PS-IAA4 (construct 30) and PS-IAA4-like proteins from *Arabidopsis thaliana* (IAA1, IAA3, IAA5, and IAA9) which comprise the invariant amino acids in BC-A and BC-B of the conserved bipartite basic motif (indicated by stippled boxes and given above or below) were tested as N-terminal GUS fusions. The extent of the peptides and number of amino acids which separate the basic elements (spacing) are given on the left.

(c) Sequence comparison of the conserved bipartite basic motif in PS-IAA4-like proteins. The sequences of experimentally tested bipartite NLS peptides (an asterisk indicates the GUS fusion site) are aligned with corresponding sequences of related proteins. Basic amino acids in PS-IAA4 and PS-IAA6 are underlined which have been shown simultaneously to be necessary for exclusive nuclear localization of the full-length protein. Corresponding invariant amino acids in tested and predicted bipartite NLS sequences are shown in bold face type. Additional basic amino acids proximal to the upstream element of the bipartite basic motif, KR, which are proposed to be part of a relatively independent SV40-type NLS, as in PS-IAA6, are also shown in bold face type. Sources of the sequences are as follows: a and b, *A. thaliana* (Abel *et al.*, 1994; IAA4 and IAA5 are encoded by *AtAux2–11* and *AtAux2–27*, respectively (Conner *et al.*, 1990; Abel and Theologis, unpublished data)); c, pea (*Pisum sativum*) (Oeller *et al.*, 1993); d, mung bean (*Vigna radiata*) (Yamamoto *et al.*, 1992); e, soybean (*Glycine max*) (Ainley *et al.*, 1988).

(Figure 5a, compare constructs 18 and 20 with 22). The functional analysis of karyophilic signals in PS-IAA6 is schematically summarized in Figure 5(b).

Primary structure of the bipartite NLS in PS-IAA4-like proteins

The basic clusters BC-A and BC-B contain basic amino acids which are invariant in PS-IAA4-like proteins (Figure 1; Abel *et al.*, 1994). We used site-directed mutagenesis to test the hypothesis that those residues (amino acids KR in BC-A, and basic residues of motif RSXRK or CSYRKK in BC-B) constitute the bipartite NLS in PS-IAA4. The mutational analysis was performed with PS-IAA4 because, unlike in PS-IAA6, both basic clusters of the bipartite structure (BC-A and BC-B) act in a mutually interdependent manner to mediate nuclear transport. To avoid the weak effect of the less-efficient SV40-type NLS in PS-IAA4 (BC-C), mutations of conserved basic amino acids in both clusters of the bipartite structure (BC-A, KKIIHGSSVVKNNNKR^{36} → KKIIHGSSVVKNNNLE^{36}; BC-B, KAKIVGWPPIRSYRK^{82} → KAKIVGWPPICSYGT^{82}) were introduced in a mutant NLS-C background (BC-C, KRLRIMK^{178} → TGLSIMT^{178}). As shown in Figure 6(a), mutation of conserved basic amino acids in either cluster (or in both) prevent nuclear accumulation of the respective GUS::PS-IAA4 fusion proteins (compare construct 17 with 27–29). Consequently, this study defines the primary structure of the bipartite NLS in PS-IAA4 as the bipartite basic motif, KR-X_{41}-RSYRK^{82}, and confirms the interdependence of both basic elements for nuclear targeting activity.

Identification of invariant basic amino acids as constituents of the bipartite NLS in PS-IAA4 immediately suggests a similar role for the respective bipartite basic motifs in

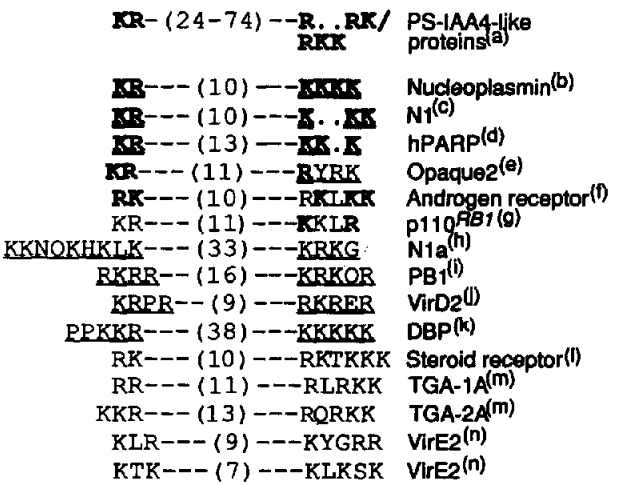


Figure 7. Comparison of functionally identified bipartite NLS sequences. Amino acids required for interdependent signal function of the basic domains are given, as identified by: series of single and multiple point mutations (bold face type underlined); sets of multiple point mutations (bold face type); progressive deletion analysis (underlined). Deduced bipartite NLS sequences which were functionally identified but not further characterized by deletion or mutagenesis are given below. Sources of the sequences are: a, this study (see reference in Figure 6); b (Robbins *et al.*, 1991); c (Kleinschmidt and Seiter, 1988); d (Schreiber *et al.*, 1992); e (Varagona and Raikhel, 1994); f (Zhou *et al.*, 1994); g (Zacksenhaus *et al.*, 1993); h (Carrington *et al.*, 1991); i (Nath and Nayak, 1990); j (Howard *et al.*, 1992; Tinland *et al.*, 1992); k (Morin *et al.*, 1989); l (Picard and Yamamoto, 1987); m (van der Krol and Chua, 1991); n (Citovsky *et al.*, 1992).

related proteins for nuclear transport. Most notably, a conspicuous feature of the bipartite motif in PS-IAA4-like proteins is separation of the discrete basic elements by an intervening region which differs considerably in size (Figure 6c). Therefore, we wished to test whether analogous sequences of selected PS-IAA4-like proteins which represent the extent of the spacer-length polymorphism (24–71 residues), can function as karyophilic signals. We constructed translational fusions between the 5′-end of the GUS coding region and sequences encoding the anticipated bipartite NLS peptides of four PS-IAA4-like proteins from *Arabidopsis thaliana* (Figure 6b and c). The parental proteins, IAA1, IAA3, and IAA9, were shown to direct GUS into the nuclei of *Arabidopsis* leaf protoplasts (Abel and Theologis, 1994; Abel and Theologis, unpublished data). The bipartite NLS of PS-IAA4 fused to the N-terminus of GUS was used as a control for nuclear localization (Figure 6b, construct 30). As demonstrated by the exclusive nuclear localization of GUS activity (Figure 6b), all peptides tested are sufficient to direct GUS to the nucleus. This suggests a general function of the bipartite basic motif in signaling nuclear targeting of PS-IAA4-like proteins.

Discussion

We have used progressive deletion analysis and site-directed mutagenesis of potential NLS sequences as complementary strategies to characterize the karyophilic signals in PS-IAA4 and PS-IAA6 as sufficient and necessary for nuclear protein import. The data reveal the function of two conserved NLS in each protein. Although their minimal sequence requirements have not been defined systematically yet, mutagenesis analysis identified an SV40-type NLS near the C-terminus, and an NLS which overlaps with the conserved bipartite basic motif near the N-terminus of each polypeptide. Both karyophilic signals belong to major classes of nuclear transport sequences for which prototypes, the NLS of the SV40 large T antigen and the bipartite NLS of the histone-binding nucleoplasmin protein from *Xenopus*, have been extensively studied (Dingwall and Laskey, 1991; Garcia-Bustos *et al.*, 1991).

The nuclear targeting sequence of nucleoplasmin is composed of two functionally interdependent basic domains separated by 10 undefined amino acids. The spacer can tolerate point mutations, small deletions and insertions (Robbins *et al.*, 1991). The nucleoplasmin-like bipartite motif is abundant in nuclear proteins and has been functionally identified as an NLS in a number of recent studies which revealed that the spacer can vary in size (see Figure 7). Spacer-length variation supports the earlier suggestion that the intervening region is only required to promote the correct, probably juxtapositional, orientation of the widely spaced basic domains specifying nuclear targeting (Robbins *et al.*, 1991). The minimal sequence of the SV40 NLS, PKKKRKV[132], was defined in great molecular detail. Although the SV40 paradigm has been successful in identifying sequences that are necessary for nuclear protein import, it has been less so in discerning sequences that are sufficient for nuclear localization (Dingwall and Laskey, 1991).

In view of (i) the shortfalls of the SV40 paradigm, (ii) the spacer-length variation in bipartite NLS sequences, and (iii) the promiscuous binding of putative NLS receptor proteins to synthetic NLS peptides of different classes (Hicks and Raikhel, 1993, 1995; Silver *et al.*, 1989; Yamasaki *et al.*, 1989), Laskey and co-workers proposed an integrated NLS consensus: both basic domains of a bipartite signal cooperate to bind a single receptor and, therefore, are interdependently required for function. However, strengthening of either domain by aquisition of additional basic amino acids would possibly allow its independent function as a monopartite signal, like the SV40 T antigen motif which is considered to be an exceptionally efficient variant of the downstream basic domain of the nucleoplasmin bipartite NLS (Dingwall and Laskey, 1991).

Our comparative study of NLS reveals a bipartite basic motif signaling nuclear import that is conserved but structurally heterogeneous in a class of ubiquitous and redundantly encoded proteins. The structural diversity of the bipartite motif in PS-IAA4 and PS-IAA6 strongly supports the proposed integrated consensus of nuclear targeting

signals (Dingwall and Laskey, 1991). The bipartite basic motif in PS-IAA4-like proteins, consensus sequence: (KKNEK)KR-$X_{(24-71)}$-(RSXRK)/(RK/RK), is not only extremely polymorphic with respect to the dimension of the spacer but is also significantly diverse regarding the amino acid composition of the upstream basic element. Both basic elements constitute the bipartite NLS in PS-IAA4, KR-X_{41}-RSYRK, and function interdependently. However, the upstream element of the conserved bipartite motif in PS-IAA4-like proteins can contain additional basic amino acids which may allow its autonomous function as an SV40-type monopartite NLS. This possibility has been demonstrated for PS-IAA6 (KKNEKKR-X_{36}-RKK) and may be similar for the polypeptides IAA5, ARG3 and GmAux22 (Figure 6c), which all belong to a distinct phylogenetic lineage of PS-IAA4-like proteins (Oeller and Theologis, 1995). Moreover, in addition to the variation of the upstream element, the amino acid composition of the downstream element is significantly altered in certain PS-IAA4-like proteins from *A. thaliana* (Abel and Theologis, unpublished data). The consequences of a strengthened (more basic) or a relaxed (less basic) downstream element for nuclear targeting activity remain to be studied.

The N-terminal NLS in PS-IAA4 and PS-IAA6 is the dominant nuclear targeting signal and is essential for efficient nuclear localization of both proteins. The C-terminal SV40-like NLS sequence, KRLRIMK, which contains the four-residue SV40-type consensus KR/KXR/K (Chelsky *et al.*, 1989), is dispensible for efficient nuclear import. However, the functionality of NLS-C, although less efficient than NLS-N, is best seen in mutant PS-IAA4 proteins containing a disabled bipartite NLS. Both independent signals are proposed to act in an additive manner. This effect is obscured by the dominance of the bipartite NLS in PS-IAA4 but becomes evident by the exclusive nuclear localization of a PS-IAA6 fusion protein with a mutated downstream element of the bipartite basic motif. The relatively independent SV40-like upstream basic element and the C-terminal SV40-type NLS contribute to efficient nuclear targeting, although either domain functions as a less effective NLS in parental full-length PS-IAA6 protein fusions. These data are reminiscent of studies on polyoma virus large-T antigen (Richardson *et al.*, 1986) and plant potyviral NIa protein (Carrington *et al.*, 1991) which identified mutually independent sequence elements that contribute equivalently to nuclear localization. A similar cumulative effect of independent, individually sufficient but in the parental context less effective karyophilic signals has been described for the maize R protein (Shieh *et al.*, 1993).

The presence of multiple targeting signals in nuclear proteins has been frequently observed (Garcia-Bustos *et al.*, 1991). Multivalency of karyophilic signals is also realized by oligomerization, for example, pentamerization of nucleoplasmin, and has been suggested to be directly proportional to the size of the cargo and to increase the rate of nuclear import (Forbes, 1992). Likewise, Citovsky *et al.* (1992) proposed that the VirE2 protein which contains two bipartite NLS sequences (see Figure 7), functions as a molecular chaperone to mediate and facilitate nuclear uptake of the large *Agrobacterium* T-complex that consists of a 20 kb unfolded T-DNA strand associated with the VirD2 protein and with about 600 molecules of VirE2. Furthermore, multiple NLS sequences potentially allow control of nuclear targeting by differential regulation of NLS activity and accessibility in response to developmental signals and environmental cues. For instance, hormone binding to the glucocorticoid receptor results in exposure of a second, more efficient NLS (Picard and Yamamoto, 1987), and phosphorylation of sequences flanking the NLS of the SV40 large T antigen can alter both the rate and the extent of nuclear protein accumulation (Jans *et al.*, 1991; Rihs *et al.*, 1991). A developmentally regulated NLS was identified in the oncoprotein encoded by the adenovirus 5 E1a gene (Standiford and Richter, 1992), and nuclear import of VirD2 and VirE2 proteins in maize and tobacco roots is dependent on the developmental stage of the tissue (Citovsky *et al.*, 1994). PS-IAA4-like proteins contain several conserved putative phosphorylation sites in the vicinity of both karyophilic signals, and the conserved βαα-motif is proposed to mediate oligomerization (Abel *et al.*, 1994). Thus, the nuclear function of these polypeptides appears to be potentially amenable to a differential regulation at the topogenic level.

Experimental procedures

Plant material

Pea seeds (*Pisum sativum* cv. Alaska) were germinated as previously described (Ballas *et al.*, 1993). Cell-suspension cultures of *Nicotiana tabacum* (line XD) were maintained according to Howard *et al.* (1992).

Plasmid construction

All plasmids were constructed by standard recombinant DNA techniques (Sambrook *et al.*, 1989). PCR was used to synthesize authentic, truncated or mutagenized cDNAs coding for PS-IAA4-like proteins (PS-IAA4, PS-IAA6 (Oeller *et al.*, 1993); IAA1, IAA2 (Abel *et al.*, 1994); IAA3, IAA5, IAA9 (Abel and Theologis, unpublished data)). The cDNAs coding for mutant full-length polypeptides were reconstructed upon ligation and subcloning of subfragments which were individually synthesized using mutagenic amplimers with appropiate restriction recognition site linkers. To generate translational fusions with the coding region of GUS, the amplified cDNA derivatives were subcloned either into the *Nco*I site (which provides the translational start codon of the GUS coding region) of pRTL2-GUS or into pRTL2-GUS/NIaΔBam (Carrington *et al.*, 1991) after removing the NIaΔBam fragment by *Bgl*II/*Xba*I or *Bgl*II/*Bam*HI digestion, respectively. The *Bgl*II site

provides the C-terminal translational fusion site to GUS, extending the GUS protein by Arg-Ser. PCR primer sequences and specifics of plasmid construction are available upon request. All plasmid constructs were verified by dideoxynucleotide sequencing (Sanger *et al.*, 1977).

Transient transformation systems

Preparation and transfection of pea protoplasts was performed according to Ballas *et al.* (1993). Tobacco protoplasts were prepared and transfected as previously described (Abel *et al.*, 1994; Howard *et al.*, 1992).

Histochemical GUS assay

Transfected protoplasts were histochemically assayed for GUS activity and stained for DNA as previously described (Abel *et al.*, 1994). Protoplasts and cell nuclei were visualized using differential interference contrast and epifluorescence optics (Zeiss Axiophot microscope), respectively. Plasmid DNAs pRTL2-GUS::VirD2 (Howard *et al.*, 1992) and pRTL2-GUS (Carrington *et al.*, 1991) were routinely used as controls for nuclear localization and cytoplasmic localization of GUS, respectively. Data were taken only when obtained with tobacco protoplast preparations which yielded efficient nuclear localization of GUS::VirD2 fusion protein in at least 90% of the transformed (stained) protoplasts. Each plasmid construct coding for GUS–auxin gene fusions was tested two to six times. Approximately 200 stained protoplasts were inspected per construct and transfection. We observed three patterns of subcellular distribution of the fusion proteins, as evidenced by the GUS reaction product: (i) nuclear (N), staining was comparable to the nuclear localization control, GUS::VirD2 (see Abel *et al.*, 1994); (ii) cytoplasmic (C), staining was comparable to the cytoplasmic localization control, GUS (note that cytoplasmic staining is less intense in regions which are primarily occupied by vacuoles); and (iii) predominantly nuclear (N>C), intense staining of the nucleus with significant but uniform staining of the extranuclear compartment.

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